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USAFSAM-TR-88-12

PHARMACOLOGICAL PROTECTION OF THE RETINA AGAINST DAMAGING LASER EXPOSURES: A FEASIBILITY STUDY

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September 1988

Final Report for Period January 1987 - December 1987

Approved for public release; distribution is unlimited.

Prepared For
USAF SCHOOL OF AEROSPACE MEDICINE
Human Systems Division (AFSC)
Brooks Air Force Base, TX 78235-5301

NOTICES

This final report was submitted by KRUG International, Technology Services Division, 406 Breesport, San Antonio, Texas, under contract F33615-84-C-0600, job order 7757-02-82, with the USAF School of Aerospace Medicine, Human Systems Division, AFSC, Brooks Air Force Base, Texas. Lieutenant Colonel Robert M. Cartledge (USAFSAM/RZV) was the Laboratory Project Scientist-in-Charge.

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S) USAFSAM-TR-88-12
6a. NAME OF PERFORMING ORGANIZATION KRUG International Technology Services Division 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION USAF School of Aerospace Medicine (RZV)
6c. ADDRESS (City, State, and ZIP Code) 406 Breesport San Antonio, TX 78216	7b. ADDRESS (City, State, and ZIP Code) Human Systems Division (AFSC) Brooks Air Force Base, TX 78235-5301
8a. NAME OF FUNDING/SPONSORING ORGANIZATION 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER F33615-84-C-0600
8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62202F	7757	02	82

11. TITLE (Include Security Classification)
Pharmacological Protection of the Retina Against Damaging Laser Exposures: A Feasibility Study

12. PERSONAL AUTHOR(S)
Cartledge, Robert M.; Glickman, Randolph D.; and Elliott, William R., III

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 87/01 TO 87/12	1988, September	37

16. SUPPLEMENTARY NOTATION
Protocol No. RZV-87-01

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Antioxidant; Laser; Light damage; Protection; Rabbit; Retina
09	03		
06	07		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Light damage to ocular tissues may occur through at least three mechanisms -- thermal, photochemical, or mechanical (photoacoustic) stress. These stress mechanisms may act singly, or in combination, depending on the intensity, wavelength, and temporal parameters of the damaging light exposure. Because some of these mechanisms involve biochemical intermediates, the possibility exists that appropriate pharmacological therapy might limit the extent of retinal light damage. This investigation determined the degree to which pharmacological pre-treatments could protect a model retina against laser light damage. Five agents were examined for protective activity: an anti-inflammatory steroid hormone (dexamethasone); three anti-oxidants (3-aminotyrosine, vitamins C and E); and a chelating agent (EGTA). Experiments were conducted in the isolated, perfused eyecup of the white New Zealand rabbit. The measure of retinal response was the transretinal ERG elicited by a white test light. The protective action of each agent was measured by the degree to which it maintained the ERG amplitude following a tissue-damaging exposure from a krypton laser tuned to 530 nm. The laser exposure

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION	
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Robert M. Cartledge, Lt Col, USAF, BSC	22b. TELEPHONE (Include Area Code) (512) 536-3622	22c OFFICE SYMBOL USAFSAM/RZV

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

UNCLASSIFIED

19. ABSTRACT (Cont'd.)

was 1 to 3 times the ED_{50} for lesion production and produced at least a 50% depression of baseline ERG amplitude. While none of the five agents completely prevented depression of the ERG, pretreatment with 500 µm 3-aminotyrosine provided statistically significant protection, particularly at higher test light intensities. The EGTA-treated retinas exhibited some resistance to the laser insult, but the degree was of borderline significance. The other agents did not provide measurable protection. Previous studies have indicated that antioxidant treatment can reduce the damage caused by chronic light stress; the present results indicate that acute light damage, too, may be ameliorated by antioxidant therapy.

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PHARMACOLOGICAL PROTECTION OF THE RETINA AGAINST DAMAGING LASER EXPOSURES: A FEASIBILITY STUDY

INTRODUCTION

Light damage to the eye, whether produced by laser exposures or by non-coherent sources, occurs through several mechanisms (see, for example, Ham et al. (1)). Light damage may occur through thermal, photochemical, or mechanical (photoacoustic) mechanisms. These processes may act singly or in combination, depending on the intensity, wavelength, and temporal parameters of the damaging light exposure. Currently, most modalities of protection against laser threats involve physical barriers (e.g., filters) to block or attenuate the laser energy entering the eye. Increasing sophistication and flexibility of laser systems, however, make infeasible the provision of complete eye protection with a transmission barrier alone. An understanding of the mechanisms through which light damage occurs may lead to additional modes of protection, for example, by leading to various pharmacological or physiological interventions which can prevent light damage to sensitive tissues. While purely mechanical damage is probably refractory to moderation through pharmacological treatments, the other two mechanisms might be ameliorated by appropriate pharmacological or biochemical intervention.

Thermal damage occurs when cellular structures are coagulated or vaporized by the deposition of heat energy. Although few biochemical pathways are known to be involved specifically in thermal effects, there is a class of "heat shock" proteins produced by biological systems, including the retina, after temperature stress. Induction of these proteins may be a way to protect against light-induced, retinal thermal damage (2). Pre-treatment with steroid hormones may also be useful in this regard (3), probably because of their anti-inflammatory action.

Photochemical damage may result from several mechanisms, e.g., peroxidation of lipids and degradation of proteins and other cellular constituents by free radicals. This type of damage is probably the most susceptible to pharmacological protection. Antioxidants, free-radical scavengers, and enzymatic reduction reactions are the most likely candidates for protective treatments (4-6). Steroid hormones have also been shown to be effective in reducing photochemical damage following retinal exposure to a broad-band light source (3).

Photoacoustic damage results when the peak power deposited during a short (<1 μs) pulsewidth exposure is greater than can be dissipated through thermal relaxation. Tissue is disrupted because of rapid physical expansion and contraction during the heating and cooling cycle. This process presumably does not involve biochemical intermediates and is therefore the least likely to respond to pharmacological modification. Reducing the peak power incident on the cornea or retina will continue to be the best protection against this particular exposure hazard.

The present project was designed to examine the feasibility of protecting the retina from the damaging effects of a laser exposure with pharmacological pretreatments. Five protective agents were tested for protective activity. These agents included three antioxidants, a chelator, and an anti-inflammatory steroid hormone. The experiments were conducted in the perfused, isolated rabbit eyecup. This system was selected because it is a mammalian retina which can be maintained in vitro for many hours (7), while being treated with pharmacological agents in the perfusion bath. Retinal function was monitored via the electroretinogram (ERG), which has already been shown to be sensitive to damaging laser exposures (8,9). Laser exposures were made with the 530.9-nm output of a krypton continuous-wave (CW) laser, with exposure durations ranging from 0.5 s up to several seconds. These exposure parameters should have activated both thermal and photochemical, but not photoacoustic, damage processes. The protection conferred by the various pharmacological treatments was measured by the degree to which they prevented a change in the ERG stimulus-response curve following the laser exposure.

METHODS

Experimental System

All experiments were performed in the excised eye of the white New Zealand rabbit (Oryctalagus cuniculi). The rabbits were all adult males weighing at least 2 kg. Prior to surgery, the animals were sedated with an intramuscular (i.m.) injection of ketamine HCl (20-30 mg). A catheter was implanted in an ear vein and Nembutal anesthesia was subsequently administered through this route. Because rabbits can easily be lethally overdosed with barbiturates, surgical plane anesthesia was achieved by administering a series of Nembutal injections, starting with an initial bolus of 25 mg (delivered in 1 ml volume of Ringer's). Additional doses were given in 0.2 ml (5 mg Nembutal) increments while the animal was being prepared for the surgery. By the time the eye was excised, the rabbit may have received up to 75 mg Nembutal, although there was significant variation between animals with respect to their tolerance to the anesthesia. Physical signs, e.g., absence of muscular activity or tachycardia following firm pedal pressure, were used to gauge when the animal was in a surgical plane of anesthesia. Following the excision of the first eye, the orbit was packed with gauze to control bleeding and the rabbit was kept deeply anesthetized until the second eye was removed for study; typically about 3 h later. The animal was continuously monitored during this time to ensure that it was adequately anesthetized. After removal of the second eye, the rabbit was euthanatized by barbiturate overdose.

The eye was excised from the rabbit as quickly as possible and placed on a piece of filter paper moistened with rabbit Ringer's solution (same as the perfusate, see later). An incision was made through the sclera at a point just posterior to where the ora serrata was estimated to lie. An

iridectomy scissors was used to complete a circumferential cut around the eye and the anterior segment was lifted away from the posterior pole (the "eyecup"). Usually the vitreous body remained behind, but because of its gelatinous nature in the rabbit, it could be gently removed without tearing the retina. Four or five radial cuts, each about 1-2 mm long, were made in the edge of the eyecup to permit it to be spread flat on the base of the perfusion chamber (retina side up). The eyecup was fixed in place by clamping the top of the chamber in position. The chamber was then placed in the optical train and connected to the perfusion system. With this technique, perfusion could be started within 4.5 min of removing the eye from the rabbit. To allow the retina to reach a stable baseline, it was allowed to dark adapt under perfusion for 45-60 min prior to commencing any recordings (see Results section).

Eyecup Perfusion Chamber

The design of the chamber was similar to that of Miller & Dacheux (10). The chamber was constructed of nylon, in two parts (Fig. 1). The base consisted of a pedestal with a convex top, over which the eyecup could be everted, retina side up. In this base were embedded two concentric silver/silver chloride (Ag/AgCl) electrodes which made contact with the scleral surface. The eyecup was held in place by an upper shell which fit over the pedestal and was clamped in place, creating a watertight seal. This upper shell contained a single Ag/AgCl electrode in contact with the perfusate flowing across the tissue. An opaque shield was fixed over this upper electrode to minimize direct exposure to the stimulus light (to avoid photoelectric artifacts). The ERGs were recorded transretinally through the electrodes in the base and upper shell. The upper shell also contained an inlet and outlet for the perfusate. Depending on the flow velocity, the depth of this perfusate was 4-4.5 mm. A thermistor in the shell monitored the temperature of the perfusate. The entire chamber was fixed to a piece of Plexiglas clamped in the movable stage of an inverted microscope which served to support the preparation in the optical system.

Recirculating Perfusion System

The perfusate was contained in a 500-ml glass container and was continually bubbled with a 95% O_2-5% CO_2 mixture. Perfusate flow was maintained by gravity and regulated at 20-30 ml/min by a variable area flowmeter (Cole-Parmer 3218-29, 136-ml max. flow rate). Before reaching the chamber, the perfusate passed through a glass coil submerged in a water bath heated to approximately 45 ^{0}C. This temperature was regulated so that by the time the perfusate reached the chamber, its temperature was about 35-36 ^{0}C. (The preparation was maintained at slightly below normal body temperature to prolong its viability.) The perfusate was drawn from the chamber by a peristaltic pump (Cole-Parmer Masterflex pump system, motor: 7553-20, pump head: 7014-21), and returned to the reservoir. Number 14 silicone composition tubing (Cole-Parmer 6411-14) was used in the pump head; elsewhere the perfusate flowed through Tygon tubing.

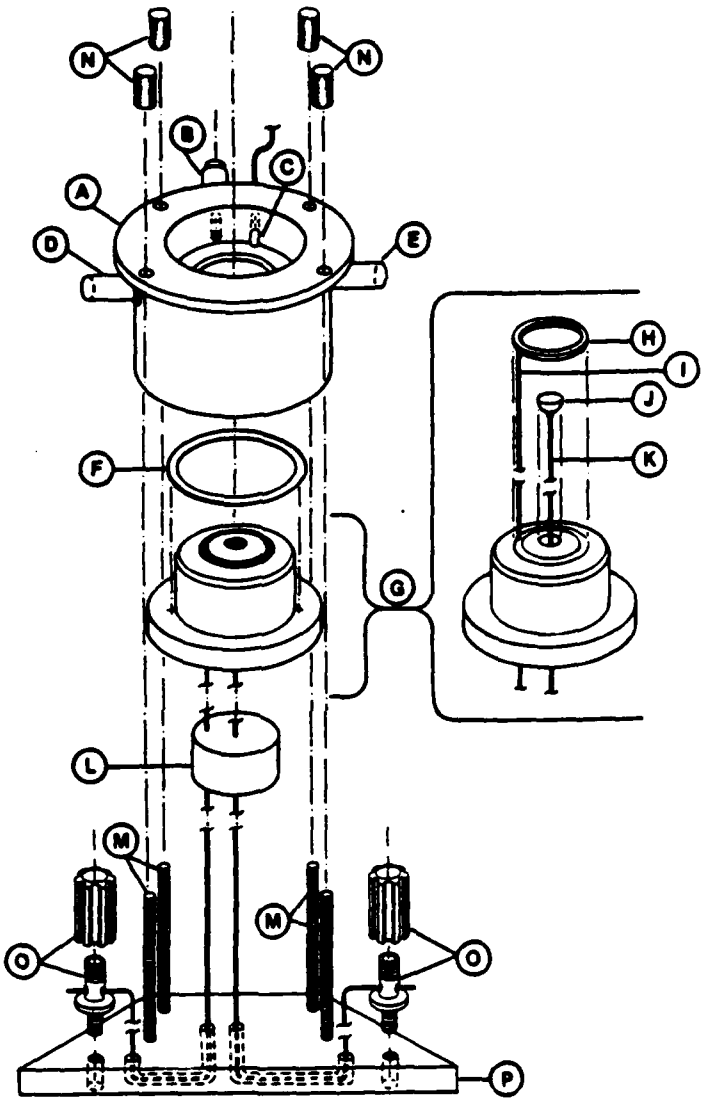

Figure 1. Exploded view of the perfusion chamber used for the eyecup preparation. Explanation of figure keys:

A - Nylon top unit, perfusion chamber
B - Upper ERG Ag/AgCl electrode
C - Thermistor
D - Perfusate inlet
E - Perfusate outlet
F - Neoprene O-ring
G - Nylon base unit, perfusion chamber
H - Base unit outer Ag/AgCl electrode
I - Connecting wire for "H"
J - Base unit center Ag/AgCl electrode
K - Connecting wire for "J"
L - Insulator, inside base unit
M - Threaded posts
N - Retaining nuts for "M"
O - Electrical binding posts
P - Plexiglas base

Composition of Perfusate

A mammalian Ringer's solution was used to perfuse the isolated rabbit eyecup. The composition of the solution is given in Table 1, and is essentially that of Ames and Nesbett (7). The solution was prepared on the day of each experiment, allowing 500 ml per retina. For convenience, stock solutions were made of all of the inorganic constituents and of all organic constituents except for ascorbate, glucose, and the serum. Dilutions were made from these stocks to obtain the working concentrations. Ascorbate and glucose were added in powder form, and aliquots of the serum were kept frozen until use. The prepared Ringer's was then gassed with a mixture of 95% O_2 and 5% CO_2 and, if necessary, the pH was adjusted to 7.4 with NaOH or HCl.

Electrophysiology

Although single unit activity was occasionally recorded from the eyecup through glass-insulated tungsten microelectrodes, the principal measure of retinal function was the ERG. The ERG was recorded differentially across the retina through the Ag/AgCl electrodes in the perfusion chamber. The upper chamber lead was the positive lead (vitreal surface of retina) and the central lead in the chamber's base was the negative lead (scleral surface). The outer, concentric electrode in the base formed the ground electrode. Signals were amplified with a Grass 7P511 preamplifier with the bandpass set to 0.3-1000 Hz. The ERGs were elicited with a 50 ms, white flash derived from a 150 W xenon arc lamp and directed down on to the retinal surface. Ultraviolet (UV) was attenuated by two glass condensers in the light path, and no infrared (IR) filter was used. The circle of retina exposed to the stimulus had a diameter of 17 mm. Flash repetition rate was approximately once per 3 s. Flash intensity was controlled by inserting a neutral density filter in the light path. The irradiance (integrated over 370-730 nm) of the unattenuated stimulus light was 11.3 $\mu W/cm^2$. Stimulus intensities are given in the figures as the log units of attenuation from maximum intensity. Electrical responses were displayed and averaged on a programmable digital oscilloscope (Norland Prowler). Typically, the ERG was measured by averaging the responses to 40 successive flashes. The ERG waveform generally exhibited a prominent b-wave with a late PII component (Fig. 2). An a-wave was generally not apparent, even with high-intensity stimuli. The c-wave was never observed, which is characteristic of albino animals (11,12). The principal measure taken from the ERG was the b-wave amplitude, measured as shown in Figure 2. This response measure (in volts) was plotted against the relative log of the stimulus intensity to construct intensity-response curves (V-Log(I) plots).

Pharmacological Agents

The five agents, and the concentrations at which they were tested, are shown in Table 2. In the cases of agents which have a clinical use,

TABLE 1. CONSTITUENTS OF RABBIT PERFUSATE

I Inorganic Constituents

Component	mg/L	mM
NaCl	7010	120
KCl	370	5
$NaHCO_3$	2100	25
Na_2HPO_4	110	0.8
NaH_2PO_4	10	0.1
$CaCl_2 \cdot 2H_2O$	290	2
$MgSO_4 \cdot 7H_2O$	250	1

II Organic Constituents

Component	mg/L	µM
L-glutamine	73	500
taurine	0.75	6
choline Cl	0.6	4.3
myo-inositol	27	150
Na pyruvate	22	200
ascorbate	18	100
glucose	1800	10,000

III Other Constituents

Component	ml/L	Comments
100X BME vit	2.5	BME vitamins, Sigma #B6891
100X MEM a.a.	2	MEM non-essential amino acids, Sigma #M7145
horse serum	5	sterile filtered, Sigma #H6762

Notes:

1. Perfusate gassed with 95% O_2-5% CO_2 mixture.
2. Final pH adjusted to 7.4 with NaOH or HCl.

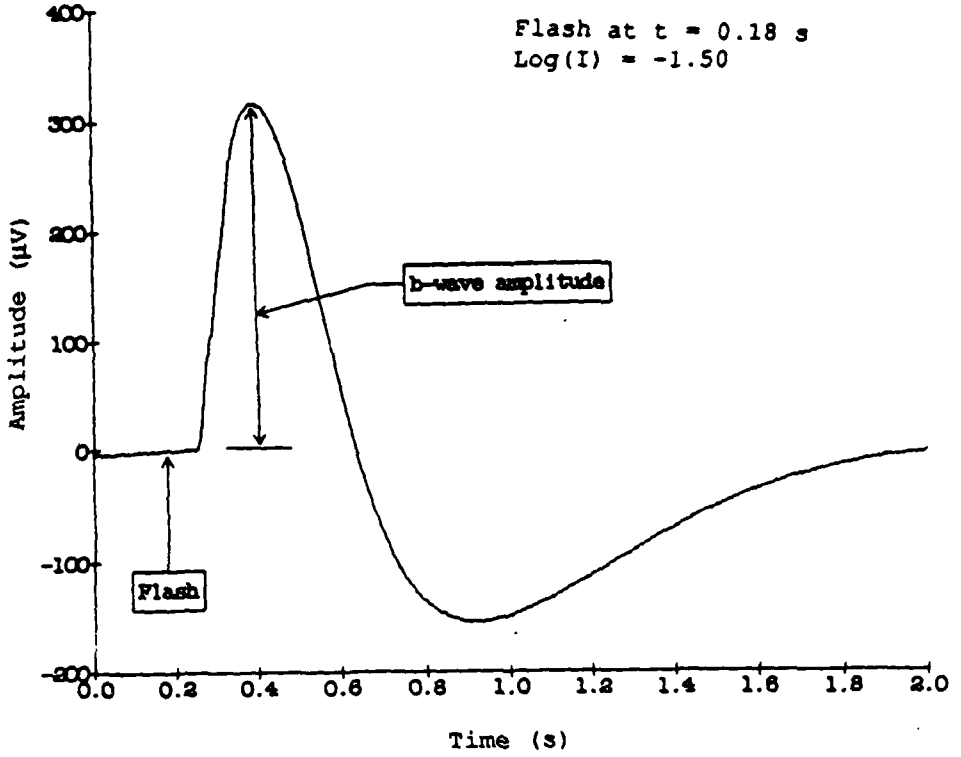

Figure 2. Method of measuring the b-wave amplitude from rabbit ERG. The stimulus was a flash of white light presented at t = 180 ms. The trace shown here is an average of 40 responses. B-wave amplitude was measured from the start of the b-wave inflection to its apparent peak.

TABLE 2. WORKING CONCENTRATIONS OF PROTECTIVE AGENTS

Agent	Concentration in Perfusate	N	Molarity	Notes	FW
Ascorbate	78 μg/ml	3	443 μM		176.1
(vit. C)	88 μg/ml	1	500 μM		
Dexamethasone	8.3 μg/ml	1	16 μM	Sodium	516.41
	9 μg/ml	5	17 μM	phosphate injectable	
d-alpha-tocopherol	0.06 IU/ml	1		Aquasol E	472.8
(vit. E)	0.11 IU/ml	3		1.4 mg/IU	
	0.13 IU/ml	2			
EGTA	38 μg/ml	5	100 μM		380.4
3-AT	67 μg/ml	1	250 μM		269.1
	135 μg/ml	4	500 μM		

primarily dexamethasone and ascorbate (vitamin C), the concentration was selected to be at the upper end of the clinically used range. The concentration of ethylene glycol-bistetraacetic acid (EGTA) (100 μM) was selected as being below the level which would conceivably interfere with neurotransmitter release. Since there is no generally accepted clinical dosage for d-alpha-tocopherol (vitamin E) (13), a level consistent with some clinical applications was used. The dosage for the experimental anti-oxidant, 3-aminotyrosine, was selected to inhibit oxidation reactions (14) without producing immunosuppressive effects (15). The agents were applied to the retina by adding them directly to the perfusion reservoir and allowing 20-30 min for entry into the retinal tissue.

Laser

Laser exposures were made with a krypton ion, CW laser (Coherent 750K in early experiments, and Coherent Innova 90K in later experiments), which was tuned to 530.9 nm. The laser output was directed at the surface of the retina through a fiber optic channel (Newport Research F-LFI). An electro-mechanical shutter controlled the laser exposure pulsewidth. Supplementary lenses were used to focus the beam on the retina into a spot 1 mm or less in diameter. Exposure intensities were measured with a Photodyne Model 66XLA radiometer with the #350 head, and ranged at the retina from 10 to 30 mW, with a pulsewidth of 500 ms. This exposure was calculated to produce a retinal energy density of 0.64 to 1.91 J/cm^2. Although we had difficulty discerning lesions on the retinal surface following a single exposure, two successive exposures (delivered within 1/2 s) did produce a noticeable white spot in the pigment epithelium. This observation indicated that the laser exposures were near the ED_{50} for the rabbit eye.

RESULTS

Characterization of the ERG

Dark Adaptation and ERG Amplitude

The ERG amplitude depends on the state of light (or dark) adaptation of the retina. Although the waveform of the intact albino rabbit photopic ERG is somewhat unconventional, e.g., the a-wave is reduced and the c-wave may be absent, the b-wave amplitude exhibits the characteristic increase in amplitude with dark adaptation. The isolated eyecup preparation used in this investigation was no different in this respect. Up to a two-fold increase in b-wave amplitude was obtained after 1-h dark adaptation. The average intensity-response curves of light- and dark-adapted ERGs and the dark/light ERG amplitude ratios are presented in Figure 3. While the time required to reach a dark-adapted state appeared to be prolonged compared to the primate retina, it is comparable to that observed in the intact rabbit eye (16).

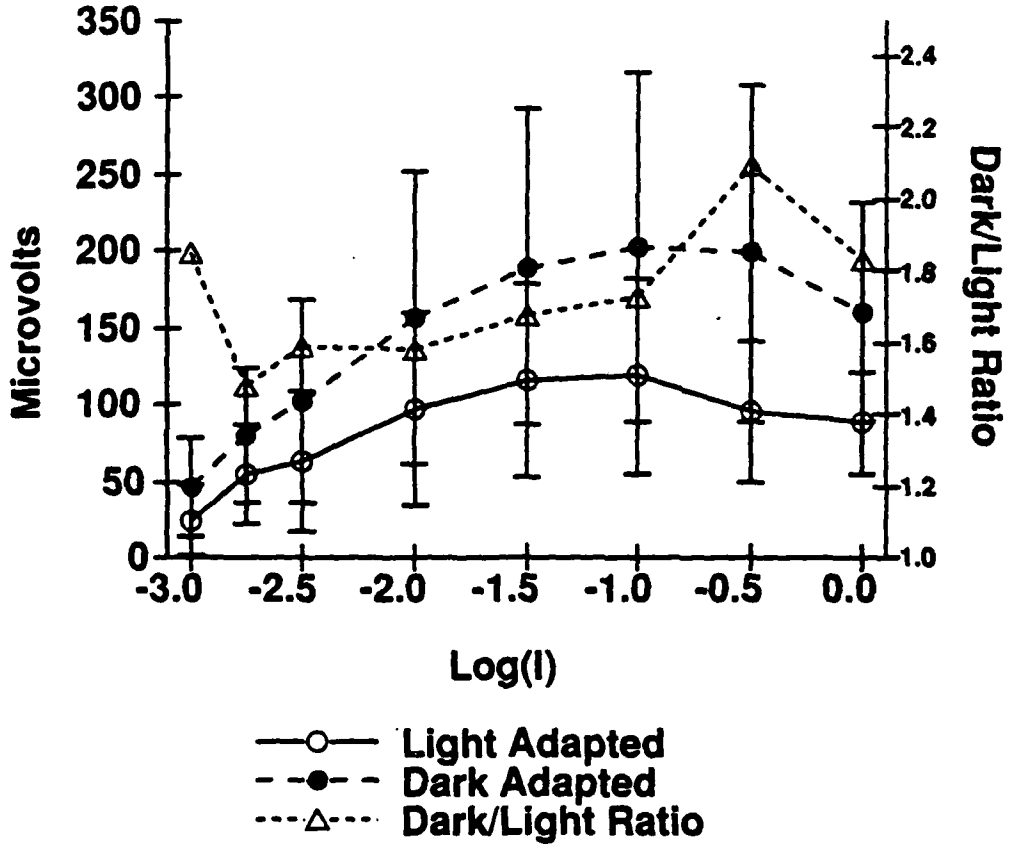

Figure 3. Increase in ERG b-wave amplitude after complete dark adaptation. Average ERG amplitude of dark- and light-adapted retinas (left ordinate) and dark/light amplitude ratio (right ordinate) are shown. Error bars indicate ±1 standard deviation.

It was important to obtain a reliable estimate of the dark adaptation time because a stable response amplitude was required in these experiments in order to observe the effects of the laser lesions and the protective agents. For this reason, all preparations were dark-adapted for a minimum of 45 min before experimentation proceeded.

It is apparent from the standard deviations shown in Figure 3 that there was considerable variability in the b-wave amplitude between retinas. The maximum ERG amplitude when the retinas were light adapted averaged 115 ±63 μV, and when dark adapted was 198 ±110 μV (cf. Fig. 3). The largest amplitude recorded was over 500 μV. The inter-eye amplitude variation was eliminated by normalizing all responses. This normalization was accomplished by expressing each ERG amplitude as a fraction of its maximum, baseline response. Consequently, the amplitude scale in all successive figures is shown as "relative response."

Intensity-Response Functions

Effects of external agents were estimated by their effect on the intensity-response or V-Log(I) curve of the retina. The curves were constructed by plotting the b-wave amplitude against the relative log of the stimulus intensity. Two sample series are shown in Figures 4 and 5. Figure 4 is the light-adapted, and Figure 5 is the dark-adapted V-Log(I) series from the same eyecup. The original records from which the b-wave amplitudes were determined are also shown in the figures. In addition to the amplitude increase during dark adaptation, the oscillatory potentials (seen on the rising phase of the b-wave) become somewhat less prominent.

Effect of Protective Pharmacological Agents

Toxicity of Agents

After the eyecup had dark adapted and the baseline V-Log(I) curve had been taken, it either received a laser exposure (laser/no protective agent), or was treated with the protective agent and then exposed (laser/plus protective agent). In the latter case, the agent was introduced into the perfusion fluid and allowed to circulate over the retina for 20-30 min. When this time had elapsed, an intensity-response curve was obtained. This curve was compared to the baseline V-Log(I) curve to determine if the agent itself had any deleterious effects on retinal response. At the concentrations used in this study (cf. Table 2), none of the five agents produced a consistent change in ERG amplitude. This lack of toxicity is illustrated in Figure 6, where, over the range of stimulus intensities used in the study, the ERG amplitude is stable before and after the application of the drug.

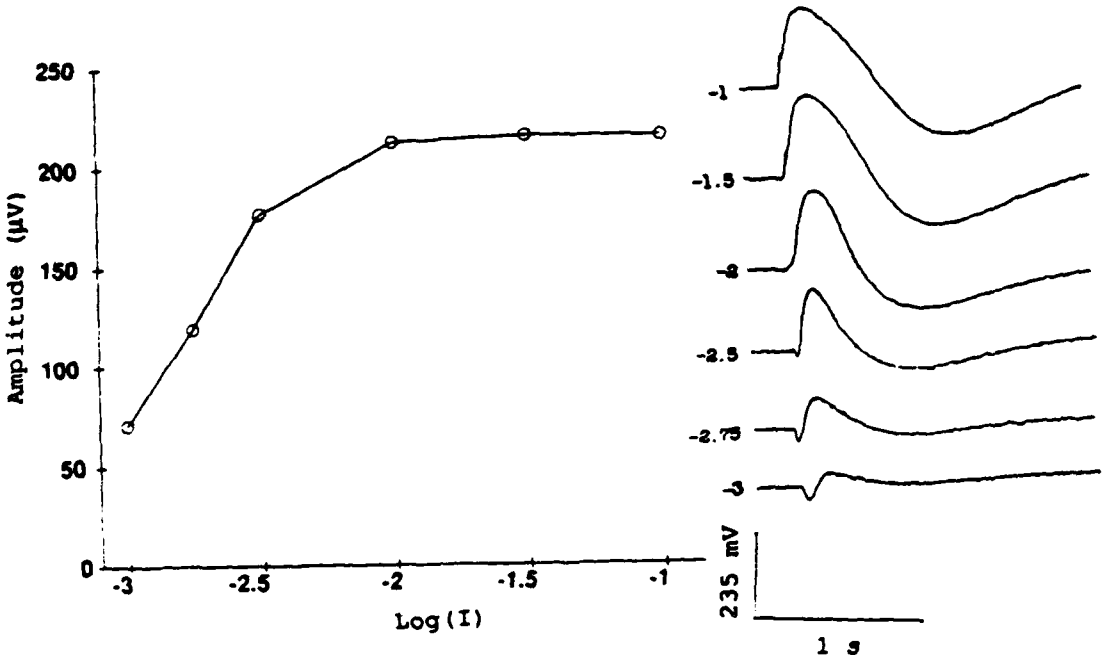

Figure 4. V-Log(I) curve of ERG recorded from light-adapted rabbit eyecup preparation. On the right are six averaged ERG responses to progressively more intense stimuli (most intense = -1). Stimulus flash presented at t = 0.18 s in all cases. The amplitude of the b-wave (prominent upward deflection) at each stimulus intensity was used to construct the curve on the left side of the figure.

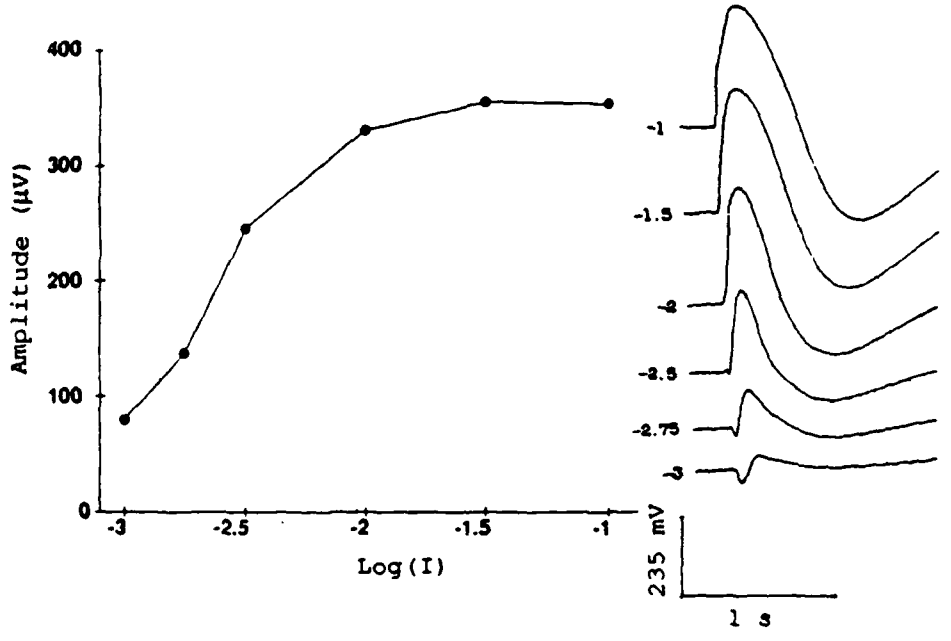

Figure 5. V-Log(I) curve of ERG recorded from dark-adapted rabbit eyecup preparation (left). Representative ERG traces shown on right side of figure. Data was collected from the same retina shown in Figure 4.

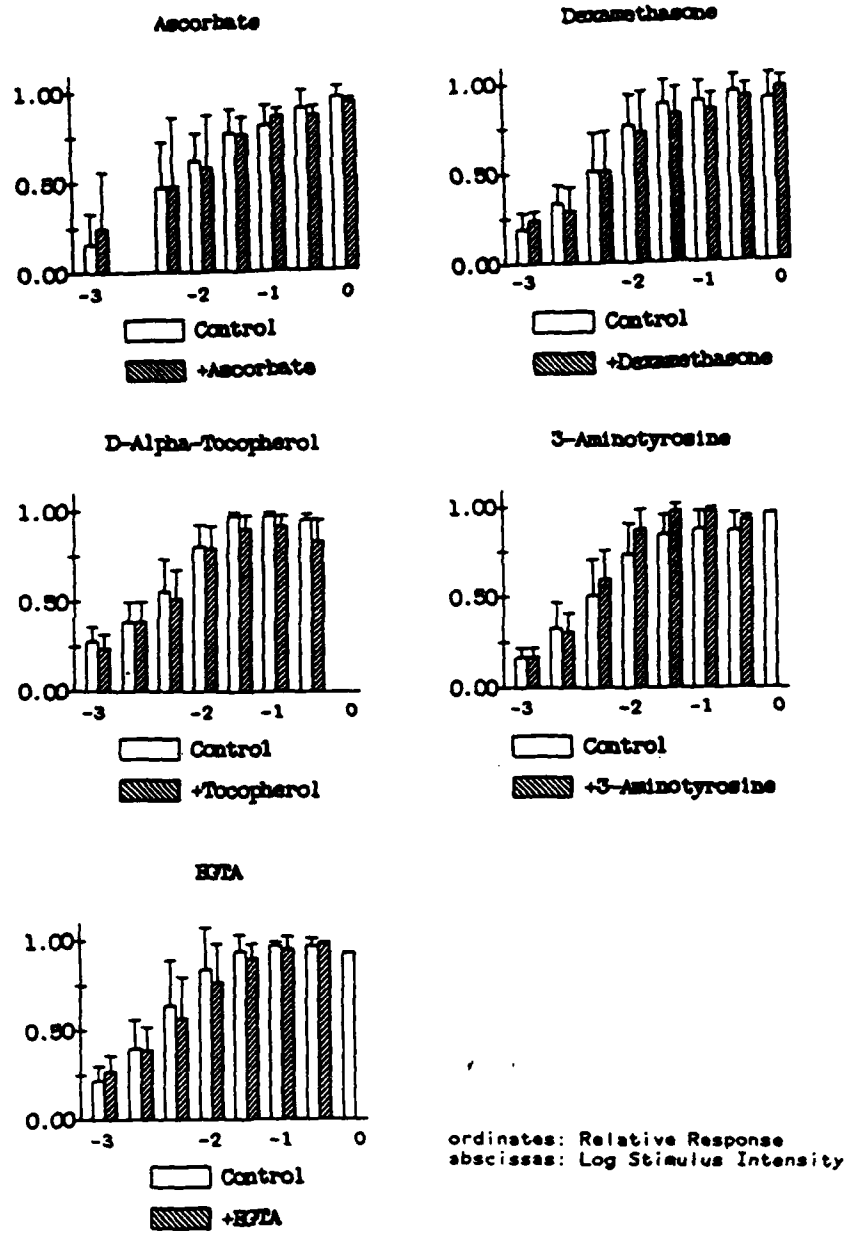

Figure 6. Lack of immediate toxicity of protective agents on the retina, as indicated by constant ERG b-wave amplitudes before and after application. Drug dosages shown in Table 2.

Effect of Laser Exposure on ERG Amplitude

Initial experiments were aimed at determining a laser exposure intensity which would reduce ERG amplitude by approximately 50%. This level was then used as the criterion exposure against which the pharmacological agents would be tested for a protective effect. With the 530.9-nm output from the krypton ion laser, the criterion level was reached with 1/2-s exposures delivering at least 10 mW to the retina. The original experimental plan was to: (1) determine baseline ERG V-Log(I), (2) make a laser exposure and determine the decrement in ERG V-Log(I), (3) add agent and determine toxic effect (if any) of agent alone, and (4) determine the decrement in the V-Log(I) curve produced by a second laser exposure and compare to step #2. This design would have facilitated analysis by generating paired drug/no drug results.

Unfortunately, this approach proved to be impractical because it required that successive laser exposures on the same eyecup preparation (made at different retinal locations) produce additive decrements in the V-Log(I) curve. This condition was not consistently met, as is illustrated in Figure 7. In the eyecup from rabbit 656, two successive laser exposures each produced roughly similar displacements in the V-Log(I) function (Fig. 7, top), i.e., the V-Log(I) curve after laser exposure #2 was displaced about as much from V-Log(I) #1 as #1 was displaced from the pre-laser curve. In the eyecup from rabbit 660, however, the second laser exposure did not significantly reduce the intensity-response curve of the retina beyond the displacement produced by the first laser exposure (Fig. 7, bottom). This displacement could not be attributed to variations in the laser output because the CW laser's output was continuously monitored. Variations in sensitivity to the laser exposure with retinal topography are a possibility, but were not explored.

The approach that was ultimately adopted was to perform laser criterion experiments in one set of retinas and protective effect studies in a separate set. The results from retinas receiving the same treatments were pooled, and unpaired comparisons of the group means were made.

The decrement in the ERG intensity-response curves produced by laser insult was determined in untreated retinas. The overall average reduction in ERG amplitude compared to the baseline response was 42%, however, there appeared to be a greater loss at lower than at higher stimulus intensities. With stimuli lower than log(I)=-2.00, the post-laser amplitude was 38% of control (62% reduction), but with brighter stimuli, it was 74% of control (26% reduction). Whether this difference reflected the ability of more intense stimuli to overcome partially the laser-induced damage, or rather, a change in the ERG gain characteristic, was not determined. These data provided the basis for comparison to the ERG decrement in laser-exposed, treated retinas.

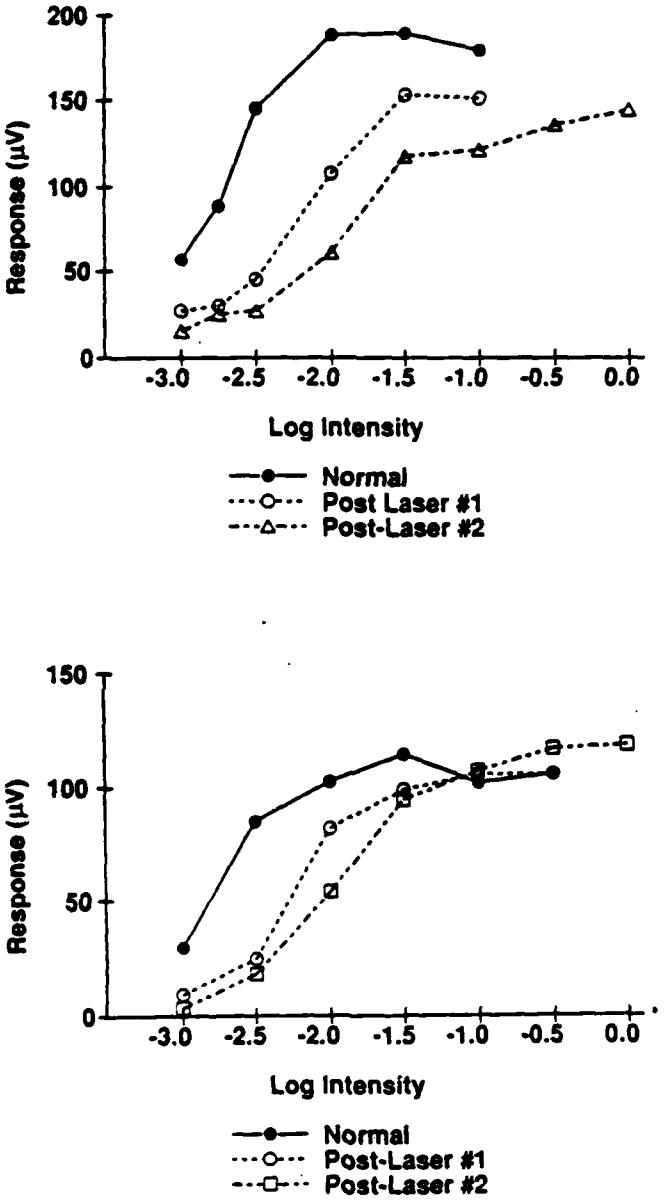

Figure 7. V-Log(I) curves showing effect of successive laser exposures on retinal response. Top: (Rabbit 656) Two successive exposures produced similar and additive decrements in ERG amplitude. Bottom: (Rabbit 660) Two successive laser exposures failed to produce additive losses in ERG b-wave.

ERG Amplitudes in Protective-Agent Treated Retinas

Intensity-response curves were obtained from retinas that had been treated for a minimum of 20 min with protective agent and then exposed to the krypton laser radiation at the criterion intensity. The responses at each of the stimulus intensities were then compared to the laser-only data obtained as described in the previous section. A summary of the means and standard deviations from each of the experimental groups is presented in Table 3. The significance of the differences between the various treatments were tested with a t-test if variances were normally distributed, or with a Mann-Whitney test for cases in which the distribution of variances appeared to differ from the normal. Where significant differences were found, the significance levels are indicated in Table 3. The average intensity-response curves based on data taken from retinas in all the conditions used in this study are shown in Figure 8.

Pretreating the retinas with any of the following agents, ascorbate (78-88 µg/ml), dexamethasone (8-9 µg/ml), or d-alpha-tocopherol (0.06-0.13 I.U./ml) did not afford any apparent protective effect against laser insult at any stimulus intensity. The EGTA (38 µg/ml) afforded marginal protection (with borderline statistical significance) at two intermediate stimulus intensities. The experimental antioxidant, 3-aminotyrosine (67-135 µg/ml), afforded significant protection at the four highest stimulus intensities. The percent protection against laser-induced damage was calculated for each agent in the following way:

$$P = \frac{R_T - R_L}{R_C - R_L} \times 100 \qquad (1)$$

where P = percent protection conferred by the agent, R_T = relative response in eyecups treated with the agent and then exposed to the laser, R_L = relative response in eyecups exposed to the laser alone, and R_C = relative response in control eyecups. The mean of the pre-treatment ERG responses from subgroups of experiments testing each individual agent did not differ significantly from the mean of all the pre-treatment responses (significance tested with 2-tailed t-test). Therefore, this grand mean was used as the measure of R_C. The percent protection conferred by each agent is shown in Table 4. In some cases, the result produced by equation 1 produced a negative percent; these values are reported as 0 in Table 4.

It is clear that only 3-AT, and to a lesser extent, EGTA, produced a consistent level of protection against the laser insult. Although the percent protection varied somewhat with the intensity of the light stimulus used to elicit the ERG, at the four light stimulus intensities, 3-AT treatment produced 80-90% protection against laser damage (cf. Table 4).

TABLE 3. SUMMARY OF RESULTS: EFFECT OF TREATMENTS ASSESSED AS FRACTION OF MAXIMUM RESPONSE

	Type of Treatment						
Log(I)	Control	Laser	Asc.	Tocoph.	Dex.	EGTA	3-AT
-3.00	0.21±0.11	0.07±0.06	0.11±0.05	0.07±0.03	0.09±0.05	0.11±0.05	0.14±0.06
-2.75	0.36±0.12	0.13±0.07	0.22	0.08±0.04	0.12±0.06	0.12±0.03	0.19±0.03
-2.50	0.54±0.22	0.24±0.17	0.12±0.09	0.20±0.12	0.16±0.07	0.26±0.07	0.36±0.06
-2.00	0.75±0.18	0.46±0.21	0.30±0.14	0.44±0.20	0.40±0.16	0.54±0.09[a]	0.69±0.05[b]
-1.50	0.89±0.13	0.64±0.15	0.54±0.19	0.57±0.22	0.64±0.22	0.72±0.10[a]	0.85±0.03[c]
-1.00	0.91±0.11	0.71±0.12	0.65±0.19	0.61±0.19	0.67±0.19	0.77±0.11	0.89±0.05[c]
-0.50	0.92±0.09	0.76±0.10	0.68±0.11	0.62±0.11	0.75±0.17	0.76±0.11	0.89±0.07[d]
N	31	11	4	6	6	5	5

Significance levels (probability of difference from laser-treated retina not significant)

[a] $p \approx 0.05$
[b] $p < 0.05$
[c] $p < 0.02$
[d] $p < 0.01$

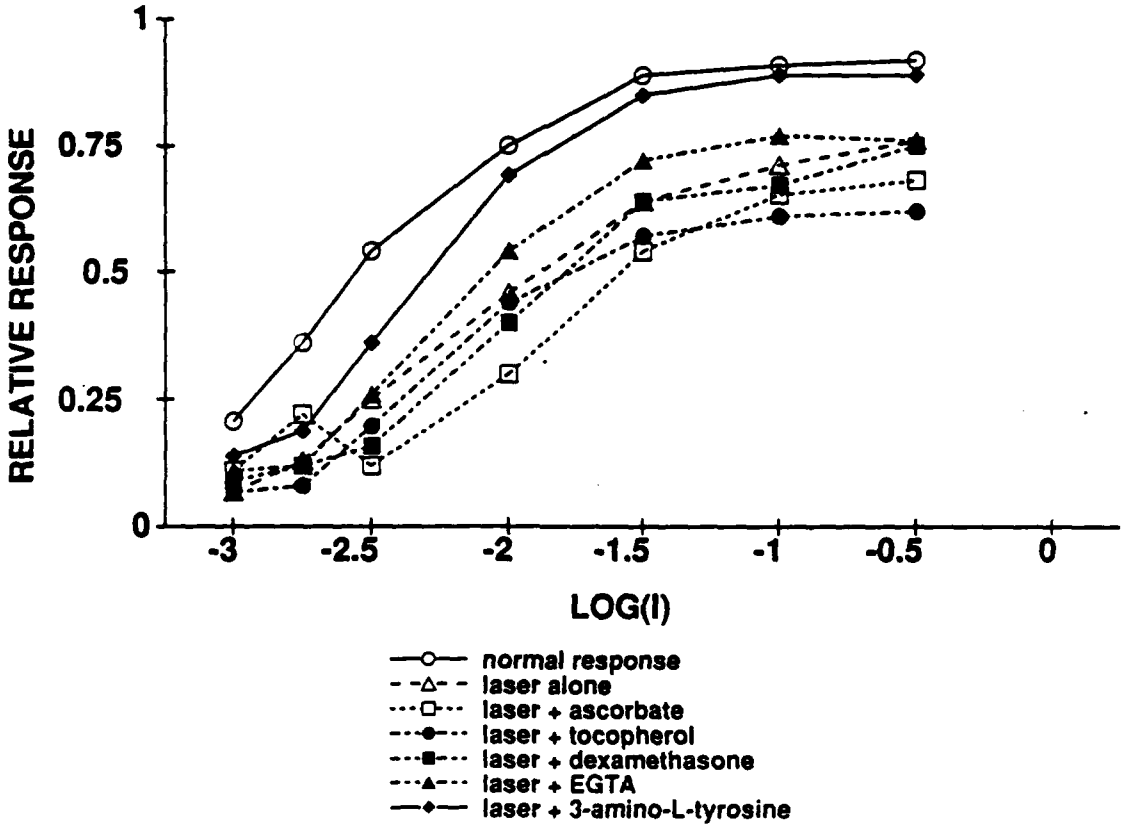

Figure 8. Summary V-Log(I) curves showing normal retinal response (unexposed, no protective treatments), the response after a single laser exposure without protective therapy, and the response functions after a single laser exposure with prior protective treatments. Points are the means of the experimental determinations. Standard deviations are shown in Table 3.

TABLE 4. PERCENT PROTECTION BY PHARMACOLOGICAL TREATMENTS AGAINST LASER DAMAGE

	LOG(I)	VIT. C	VIT. E	DEX	EGTA	3-AT
	-3.00	28.6	0.0	14.3	28.6	50.0
	-2.75	39.1	0.0	0.0	0.0	26.1
	-2.50	0.0	0.0	0.0	3.4	37.9
	-2.00	0.0	0.0	0.0	27.6	79.3
	-1.50	0.0	0.0	0.0	32.0	84.0
	-1.00	0.0	0.0	0.0	30.0	90.0
	-0.50	0.0	0.0	0.0	0.0	81.3
MEAN		9.7	0.0	2.0	17.4	64.1
ST DEV		15.6	0.0	5.0	14.1	23.7

In terms of maintenance of retinal sensitivity following the laser exposure, pretreatment with 3-AT provided about 0.5 log unit of protection. Figure 9 illustrates how this determination was made. The uppermost curve shows the baseline (unexposed) V-Log(I) curve. The lowest curve (dashed line) shows the V-Log(I) curve obtained from untreated retinas exposed to the laser. The middle curve shows the V-Log(I) curve from treated, laser-exposed retinas. The horizontal line drawn at relative response = 0.5 indicates, for each of these three conditions, the stimulus intensity required to elicit a half-maximal response. The stimulus required to reach this criterion level was about 0.5 log unit less intense for the 3-AT treated retinas than for the untreated, laser-exposed retinas, indicating that the 3-AT treatment preserved some retinal sensitivity after the laser insult. The 3-AT treated retinas were only about 0.25 log unit less sensitive than the unexposed control retinas.

Effect of Protective Agents on Recovery Rate Following Laser Flash

The lengthy time required for the rabbit retina to become dark adapted made it predictable that, after the laser flash, the retina would require several minutes to readapt to the preflash level of sensitivity. As noted in the Methods section, to ensure that the retina had returned to a steady state of dark adaptation after the laser flash, 30 to 40 min were allowed to elapse after the laser exposure before a V-Log(I) curve was determined. During the postflash recovery time, the recovery of the retina was monitored with a -2.00 log flash. Although the question of laser effects on the dark adaptation rate had not been included in the original scope of the project, it seemed likely that the measurements obtained during the post laser-flash recovery period might provide at least a partial answer to this question.

Because of limited time resolution of the post-flash data (i.e., the b-wave amplitude was measured at 3-min intervals after each 40-flash average was completed), a regression line was fit to the available data points and extrapolated back to the time of the flash. The assumption was made that immediately after the laser exposure, the ERG amplitude was zero. This observation was qualitatively confirmed by inspections of the oscilloscope display of the raw data during the experiment. In most cases, no ERG was observed for 3 to 4 stimulus flashes (10-14 s postflash). Since the amplitude of the ERG should increase with first order kinetics, a Michaelis-Menton first order equation, in the form of a hyperbolic tangent function, was fit to the data (17):

$$V = A * (1 + \tanh(t - c)) \qquad (2)$$

where V = the ERG amplitude at time t, A is the half-maximal amplitude ($V_{max}/2$), t is the time (in seconds) after the laser flash, and c is the time at which $V = V_{max}/2$. V is expressed as a fraction of maximum response. Curves were fit to the data points obtained from experiments in which the laser was used alone, as well as when protective agents were applied. The family of curves generated is shown in Figure 10. It appears that with the

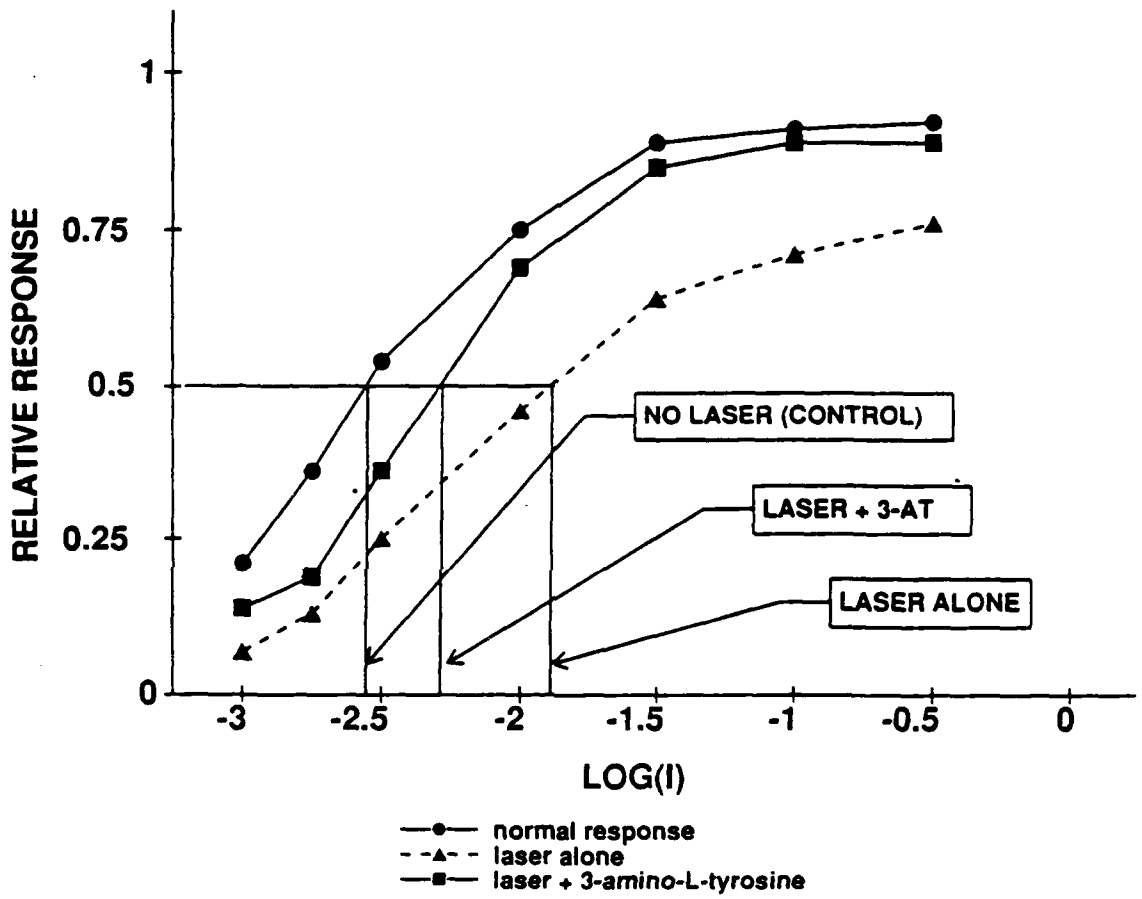

Figure 9. Analysis of protective effect of 3-AT in terms of retinal sensitivity to light stimulation. These three curves were extracted from those shown in Figure 8. See text for details.

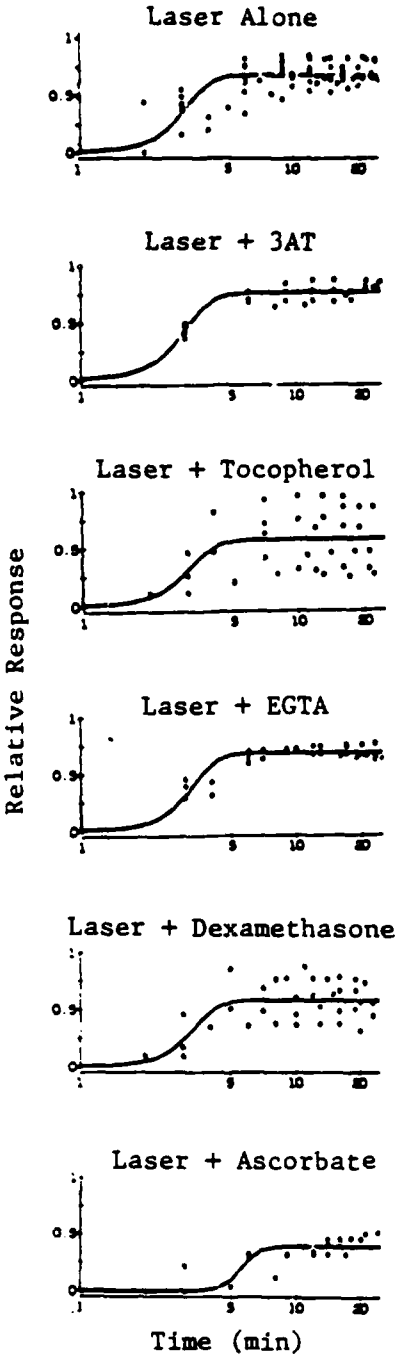

Figure 10. Rate of recovery after laser flash: effect of protective treatment. ERG amplitudes to a Log(I) = -2.00 flash were determined after laser exposures with and without protective treatments. Regression lines are hyperbolic tangent functions fitted to the experimental points (see text for explanation).

exception of the retinas treated with ascorbate, the recovery rates were very similar. A more quantitative assessment can be made by examining the equations of the regression lines, which directly yield the time to half-maximal recovery as well as the amplitude of the half-maximum response. These data are shown in Table 5 and indicate that none of the protective agents produces any real improvement in recovery time. Time to recover half-maximum ERG amplitude is about 3 min, and to 99% recovery is 6 to 6.5 min. The worst case is after ascorbate treatment: time to half-maximal recovery is delayed to nearly 6 min, and time to 99% recovery is over 11 min.

Effect of Combining Protective Agents

In two experiments, EGTA and dexamethasone were combined to determine if an enhanced protective effect could be obtained. While the results produced insufficient data on which to base any firm conclusions, they are presented here as a basis for possible future investigations. The EGTA (100 µg/ml) and dexamethasone (9 µg/ml) were added together and allowed to perfuse two retinas for 20 min. The laser exposures were made and the resulting V-Log(I) curves were obtained in the customary way. Baseline and post-laser curves from these two retinas are shown in Figure 11. Following the laser, the ERG amplitude for low-intensity stimuli (Log(I) < -2.00) was 29% of the control, and 64% of control for high-intensity stimuli (Log(I) ≥ -2.00) (Table 6). These observations represent about the same degree of loss as when the agents were applied singly. For these two agents, at least, there was no indication of a synergistic action when applied simultaneously.

DISCUSSION

While it is possible that the isolated eyecup preparation, lacking some protective factor in the blood stream or ocular media, is more susceptible to light damage than the intact eye is, previous studies using the intact rabbit eye as a model for laser bioeffect investigations have also demonstrated that the b-wave of the ERG was reduced by single laser lesions (8,9). Those studies used a ruby laser with different output characteristics (694.3-1000 nm, 800-µs pulsewidth) than the krypton CW laser used in the present study. The lesions resulting from the ruby laser exposures were probably due primarily to thermal processes (because of the ruby laser's relatively long wavelength), while the 530.9-nm, 500-ms exposures used in this investigation produced damage through both thermal and photochemical mechanisms (1). Nevertheless, the retinal irradiances involved in this study (0.64 to 1.91 J/cm^2) were comparable to those used by the previous workers; Hempel (8) reported 0.9-6.3 J/cm^2, while Priebe & Welch (9) reported 0.45-3.2 J/cm^2. The exposure intensities used in our work were 1 to 3 times the lesion threshold reported for the rabbit retina by Clarke (18).

TABLE 5. EFFECT OF PROTECTIVE AGENTS ON RECOVERY KINETIC AFTER SINGLE LASER EXPOSURE

	$V_{max}/2$[a]	T_o[b]	T_{99}[c]
None	0.339	2.99	6.0
Ascorbate	0.197	5.57	11.1
Tocopherol	0.284	2.94	5.9
Dexamethasone	0.292	3.21	6.4
EGTA	0.342	3.11	6.2
3-AT	0.377	2.85	5.7

Notes:

[a]Amplitude at half-maximal recovery expressed as a fraction of the pre-exposure maximum.
[b]Time in seconds to half-maximal recovery time
[c]Time in seconds to > 99% recovery (equals 2 x T_o)

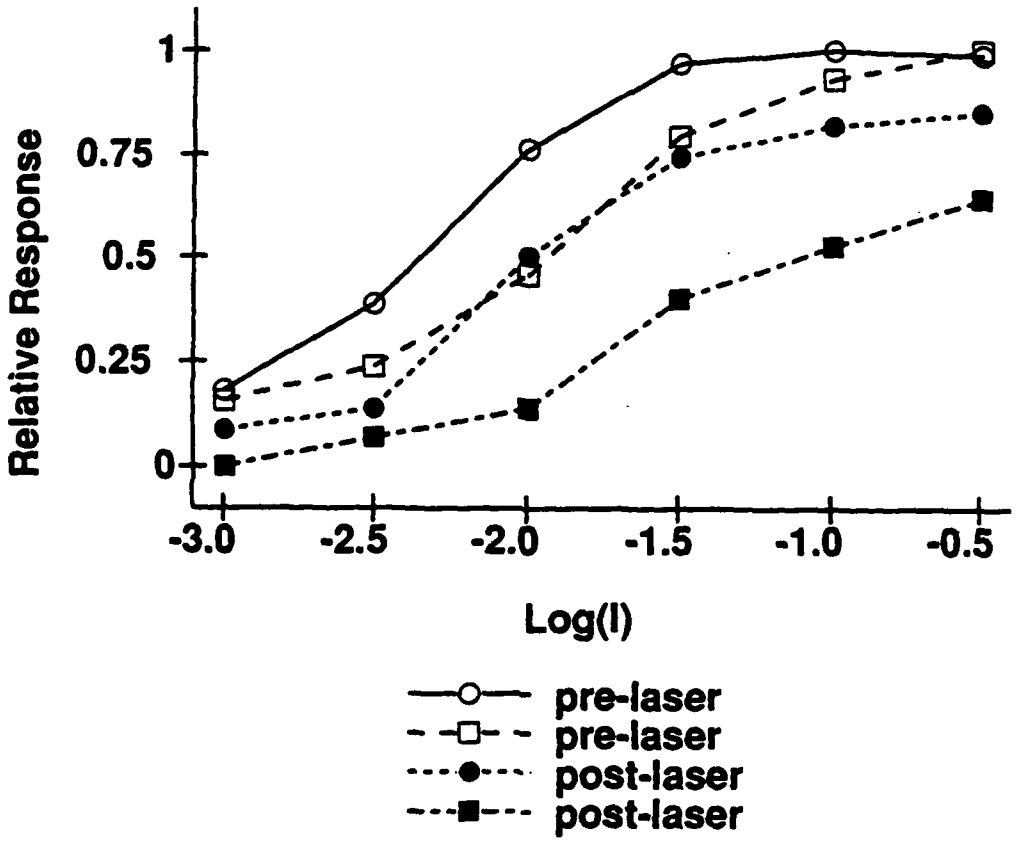

Figure 11. Effect of laser exposure on retinas treated with EGTA and dexamethasone. Open symbols are the pre-laser V-Log(I) curves from two retinas. Filled symbols are V-Log(I) curves from the same retinas after laser exposure. This combination of agents did not provide more protection than did each agent alone.

TABLE 6. EFFECT OF EGTA 100 μM AND DEXAMETHASONE 9 μg/ml COMBINED*

Rabbit ID	-3.00	-2.75	-2.50	-2.00	-1.50	-1.00	-0.50	Condition
716	0.18	0.17	0.39	0.76	0.97	1.00	0.99	Pre-laser
718	0.16	0.18	0.24	0.46	0.79	0.93	1.00	Pre-laser
716	0.09	0.06	0.14	0.50	0.74	0.82	0.85	Post-laser
718	0.00	0.00	0.07	0.14	0.40	0.53	0.64	Post-laser
	Percent ERG Amplitude Remaining After Laser Exposure							
716	50	35	36	66	76	82	86	
718	0	0	29	30	51	57	64	
			29%			64%		
			(Avg. low stimulus intensity ERG amplitude)			(Avg. high stimulus intensity ERG amplitude)		

*Normalized data

The real significance of the present study depends on the mechanism by which a restricted laser exposure (approximately 0.8 to 1.0 mm in diameter) is able to reduce the amplitude of the ERG b-wave, which is an evoked potential reflecting activity over the entire, stimulated portion of retina and pigment epithelium. With the perfusion chamber used in these experiments, there was approximately 227 mm^2 of retinal surface available; the stimulating light covered this area completely. Therefore, the lesioned area represented less than 0.5% of the stimulated retina, yet the ERG amplitude was reduced over 50% in some cases. This decrease indicated that the laser exposure triggered some global process which affected the ERG. A trivial explanation, such as light adaptation produced by scattered light, may be ruled out because the decrement in ERG amplitude was stable, i.e, there was no indication of any recovery during the 1 to 2 h the experiment was conducted following laser exposure.

The ERG amplitude could be reduced by a shunting pathway created in the retinal pigment epithelium (RPE) by the laser lesion; this shunt would reduce the normal transretino-scleral resistance, hence reducing the effective ERG amplitude. Such a mechanism was proposed for the nonlinear relationship between the fraction of retina remaining and the ERG amplitude (expressed as a percent of normal controls), in rats with light-induced retinal damage (19). This type of damage was characterized by complete loss of photoreceptors and RPE cells, so that a shunt pathway in the injured regions was possible. More recent studies of the effects of laser and noncoherent light damage on the ERG have both supported (20) and discounted (21) the role of changing transretinal resistance on ERG amplitude.

The demonstration in our study, that the laser-induced loss in ERG amplitude could be minimized with anti-oxidant therapy, suggests that an oxidative reaction was the cause of the decline in ERG amplitude. According to this hypothesis, an oxidative reaction (or reactions) was (were) initiated by the laser exposure and spread throughout the tissue. This reaction, or its products, caused deterioration in retinal function that was ultimately manifested in loss of b-wave amplitude. There is considerable evidence that light- and chemical-induced peroxidation reactions occur in the retina with damaging effects.

Lipid peroxidation can be initiated in biological tissue by the action of oxygen radicals, e.g., the superoxide anion (O_2^-), on lipid-containing structures such as membranes (22). This species is unstable in an aqueous environment and rapidly dismutates to hydrogen peroxide:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$

Although hydrogen peroxide itself reacts sluggishly with biological molecules (22), it can generate the highly reactive hydroxyl radical, $OH^\cdot$, through the well-known iron-catalyzed, Haber-Weiss reaction:

$$O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2} \qquad (4)$$

$$H_2O_2 + Fe^{+2} \rightarrow HO^\cdot + OH^- + Fe^{+3} \qquad (5)$$

Overall reaction:

$$O_2^- + H_2O_2 \xrightarrow[Fe^{+2}]{} HO^\cdot + OH^- + O_2 \qquad (6)$$

Since iron is a constituent of many enzymes and other proteins, this series of reactions may occur wherever superoxides are formed. Singlet oxygen is another possible oxidant that may be of concern. Normally not found in biological systems (23), singlet oxygen can be produced by photochemical exposures or photosensitizing conditions which cause spin reversal of oxygen's valence electrons (24,25). Singlet oxygen is a highly reactive oxidant and may be a consequence of intense light exposures, particularly at shorter wavelengths.

The retina, being a particularly membrane-rich organ, is very susceptible to damaging lipid peroxidation, possibly caused by each of the mechanisms previously described. Lipid peroxidation reactions can be initiated in the retina by intense or prolonged light exposure (26-28). Experimental models of lipid peroxidation have often utilized the Haber-Weiss reaction to cause lipid peroxidation (6,29). These reactions particularly affect the photoreceptors. The following damage modalities have been described: damage to the rod disk membranes (6); impaired release of photoreceptor transmitter (6); impaired ability of rhodopsin to regenerate itself (29); and increased photoreceptor plasma membrane permeability (30). Lipid peroxidation of long chain, polyunsaturated fatty acids leads to production of lipid hydroperoxides, and ultimately to aldehyde breakdown products, particularly short dienes such as malonaldehyde ($O{=}CH{-}CH_2{-}HC{=}O$) (31). These products themselves are toxic when directly applied to the retina (32).

Following the development of lipid peroxidation, the ERG amplitudes recorded from affected retinas decline (33-35). There is some evidence that albino animals (rats and rabbits) are more susceptible to peroxidative damage than are pigmented animals (32,36,37). This difference could be due to a protective effect conferred by melanosomes in the retina, since the other natural anti-oxidant protective systems (superoxide dismutase, glutathione peroxidase, vitamin E content) are approximately the same in albino and pigmented animals (36,38).

Reduction of some of the damaging effects of light by preventing the onset of peroxidation has been demonstrated in some model systems. Application of the antioxidants d-alpha-tocopherol and butylated hydroxy-toluene (BHT) (6), ascorbic acid (39), taurine and beta-alanine (4,5), have all been shown to be effective to varying degrees in reducing light-induced peroxidation, maintaining retinal structure, or preserving retinal function as measured electrophysiologically. In the present study, only one antioxidant, 3-AT, was found to be effective in preventing damage caused by acute light stress. This agent has a complex mode of action, serving as an antioxidant at lower concentrations (<1.5 mM) (15), but acting as an immunosuppressive at higher concentrations (16). Theoretically, the protective action of 3-AT should resemble that of d-alpha-tocopherol, another phenolic compound. For example, both agents should protect against lipid peroxidation by donating a proton to the lipid radical, thereby stopping propagation of the reaction:

$$L{-}O{-}O^{\cdot} + Phe{-}H \;\longrightarrow\; LH + Phe^{\cdot} \qquad (7)$$

where L represents a lipid and Phe represents a phenolic compound. The ineffectiveness of vitamin E against the laser insult in the perfused rabbit eyecup may have been due to the difficulty in getting a soluble form of the agent into the perfusate. The polysaccharide-suspended form used (Aquasol E) may not have been effective in reaching critical retinal sites. However, vitamin E has not always been found to be effective against oxidative damage (40). In a spinal cord model, the free-radical scavengers disulfiram, propyl gallate, and promethazine, were 11,000 times more effective in preventing lipid peroxidation than was tocopherol (41). Apparently, hydrophilic 3-AT represents a type of agent that is not only easier to work with than the waxy tocopherols, but also may be a more effective antioxidant. In view of the present results, further investigation of antioxidants as laser protective agents is warranted. Related investigations should also examine other systems that contribute to the removal of reactive oxygen species, e.g., superoxide dismutase and glutathione peroxidase. Pharmacological enhancement of these systems may confer additional protection against retinal light damage.

The failure of ascorbate to provide any degree of protection is somewhat surprising, in view of the results reported by Li et al. (39). Indeed, the decrement in the ERG amplitude was the greatest, and the recovery rate the slowest, after a laser exposure in the ascorbate treated retinas. It is possible that this potentiation of the laser effect resulted from elevation of ascorbate activity to the point at which Fe^{+3} ions were reduced to Fe^{+2}, which could then catalyse Haber-Weiss reactions (22).

We were not able to reproduce the findings of Parver et al. (3), who demonstrated dexamethasone could prevent some aspects of light-induced retinal damage. In that study, the animals were pretreated with the steroid for 4 months. Conceivably, the protective effect was related to a long-term hormonal effect on retinal and RPE cells. We did not observe a transient increase in ERG amplitude following steroid application on the retina, as reported by Koizuma and Honda (42).

The chelating agent, EGTA, was the second most effective protective agent, although the effect was of borderline significance. The beneficial effect of this agent might be due to either: (1) chelation of free Fe^{+2} ions, thereby preventing catalysis of the Haber-Weiss reaction, or (2) chelation of Ca^{+2} ions, which may be involved in the cross-linking of radiation and peroxidation-damaged proteins. Oligopolymerization of peroxy radical-damaged retinal proteins has been described (43). It appears worthwhile to investigate other chelators or modes of chelation as protective protocols, perhaps as an adjunct to antioxidant therapy.

It remains to be determined rigorously if the decline in the ERG amplitude after acute laser injury is due to loss of integrity of the RPE or to the effects of widespread peroxidation reactions. The efficacy of anti-oxidant therapy reported here and in other studies points to the importance of the latter mechanism. The rapid onset of the ERG deterioration also points to a biochemical involvement, as the light-induced damage reported by

Noell (19) took hours or days to develop. Nevertheless, compromise of the RPE integrity cannot yet be ruled out. A sufficiently intense laser exposure (particularly at short pulsewidths which disrupt tissue) could create an immediate hole in the RPE. While this type of exposure was not used in the present study, without histological evidence, an RPE hole cannot be discounted. Future research into pharmacological protection against laser eye damage should attempt to characterize the damage mechanisms involved. This investigation could be accomplished by performing histological examinations of the lesions, as well as assaying for evidence of peroxidation reactions, e.g., the presence of aldehydes, following the damaging exposure.

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